Research

Sensory responsiveness and the effects of equal subjective rewards on tactile learning and memory of honeybees

Ricarda Scheiner,[1,3] Anthea Kuritz-Kaiser,[1] Randolf Menzel,[2] and Joachim Erber[1]

[1]*Technische Universität Berlin, Institut für Ökologie, FR 1–1, D-10587 Berlin, Germany;* [2]*Freie Universität Berlin, Institut für Biologie, D-14195 Berlin, Germany*

In tactile learning, sucrose is the unconditioned stimulus and reward, which is usually applied to the antenna to elicit proboscis extension and which the bee can drink when it is subsequently applied to the extended proboscis. The conditioned stimulus is a tactile object that the bee can scan with its antennae. In this paper we describe the quantitative relationships between gustatory antennal stimulation, gustatory proboscis stimulation, and tactile learning and memory. Bees are 10-fold more responsive to sucrose solutions when they are applied to the antenna compared to proboscis stimulation. During tactile conditioning, the sucrose solution applied to the proboscis determines the level of acquisition, whereas antennal input is of minor importance. Bees differing in their gustatory responsiveness measured at the antenna differ strongly in their tactile acquisition and memory. We demonstrate how these differences in tactile acquisition and memory can be greatly reduced by calculating equal subjective rewards, based on individual gustatory responsiveness.

Learning in animals depends on many factors including the salience of the conditioned stimulus (CS) and the strength of the unconditioned stimulus (US) (Rescorla and Wagner 1972). Even under controlled laboratory conditions, individuals show variance in the rate of acquisition, the asymptote of acquisition, and in retention (Scheiner et al. 2001a,b, 2003; Matzel et al. 2003; Hedden and Gabrieli 2004; Dellu-Hagedorn 2005). Multiple intrinsic factors can contribute to these behavioral differences. Some of these factors may be related to individual differences in evaluating CS and US (Scheiner et al. 1999; Chester et al. 2003). These differences could reflect genetic heterogeneity at the individual level.

Studies in honeybees (*Apis mellifera* L.) can be very useful to identify important factors leading to inter-individual learning differences and their potential sources of control. Associative learning plays an important part in honeybee behavior. Bees learn very fast the location of a foraging site and the numerous characteristics of reward-yielding plants (for review, see Menzel and Müller 1996; Giurfa 2003). Individual bees differ in their foraging activities. They specialize in collecting pollen, water, nectar, or propolis (Winston 1987; Seeley 1995). The genetic background of a colony has an influence on the preferences for collecting nectar or pollen (Page and Fondrk 1995; Page et al. 1995). It has been demonstrated that bees that forage for different nutrients also differ in their sensory responsiveness for gustatory stimuli. Bees collecting pollen or water are very responsive to sucrose stimuli applied to the antenna. They display the proboscis extension response (PER) when their antennae are stimulated with low concentrations of sucrose or even with water. Nectar foragers, on the other hand, are, on average, less responsive and only show the PER at higher sucrose concentrations (Page et al. 1998; Pankiw and Page 1999; Scheiner et al. 2001b, 2003). Pollen and nectar foragers also differ in acquisition and retention in tactile and olfactory conditioning under laboratory conditions (Scheiner et al. 2001b, 2003). All of these experiments demonstrate significant correlations between gustatory responsiveness and learning in honeybees.

To analyze associative learning in honeybees, experiments under controlled laboratory conditions have proved very efficient. Under these conditions, bees learn easily to associate an odor (for review, see Menzel 1990, 2001; Menzel and Müller 1996) or a tactile stimulus with a sucrose reward (Erber et al. 1998). In the tactile learning paradigm, which we used in the following experiments, a bee learns to associate a small plate within the reach of its antennae with a sucrose reward. The bee is first allowed to scan the plate with its antennae for ~3 sec. During antennal scanning the proboscis extension response is elicited by applying a small droplet of sucrose to one antenna. After proboscis extension the animal is rewarded with sucrose, which is applied to the proboscis (Erber et al. 1998). After three to four conditioning trials, ~80% of the bees respond with the conditioned PER when the plate is presented without a sucrose stimulus (Erber et al. 1998; Scheiner et al. 1999). The rate and the asymptote of tactile acquisition are similar for tactile objects that differ in shape, surface structure, or position, provided the object has a size of ~3 × 4 mm (Erber et al. 1998).

In associative learning paradigms with bees that use sucrose solution as reward, it is generally assumed that a high sucrose concentration, such as 30% sucrose, has the same incentive reward value for all bees that respond with proboscis extension when their antennae are stimulated with that solution. Several experiments, however, recently showed that even a high sucrose concentration of 30% can lead to a very different level of acquisition in individual bees (Scheiner et al. 1999, 2001a,b,c, 2003). The reason for these learning differences probably lies in individual differences with respect to the perception and evaluation of the sucrose stimulus used as US.

These findings suggest that gustatory responsiveness is an indicator of the incentive salience of the sucrose reward in associative PER learning. Whether gustatory responsiveness as measured by antennal sucrose stimulation shows significant correlations with acquisition and retention in animals differing in gus-

[3]Corresponding author.
E-mail Ricarda.Scheiner-Pietsch@TU-Berlin.de; fax 49 30 314 73 177.
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tatory responsiveness needs to be examined. Our hypothesis that responsiveness to gustatory antennal stimulation is a robust indicator of sensory sensitivity for different modalities is supported by other studies that demonstrated that gustatory responsiveness is positively correlated with sensitivity to odors, light stimuli, and pollen (Scheiner et al. 2004). Here we test whether gustatory responsiveness correlates with the incentive salience of sucrose as expressed in acquisition and retention in tactile learning.

The relationship between responsiveness to gustatory stimulation of the antenna and gustatory stimulation of the proboscis can be directly measured in the same individuals. The hypothesis that antennal sucrose stimulation during conditioning has a larger effect on learning performance than proboscis stimulation during conditioning can be tested by using bees with the same responsiveness to gustatory antennal stimulation. These bees are stimulated with different sucrose concentrations at antennae and proboscis during tactile conditioning.

In addition, we test whether it is possible to estimate sucrose concentrations that have the same reward value for bees with different gustatory responsiveness as measured by antennal stimulation. We hypothesize that the estimated sucrose concentrations represent equal subjective rewards in animals with different gustatory responsiveness. Individuals with high gustatory responsiveness will consequently be rewarded with a lower sucrose concentration than bees with a lower responsiveness. These quantitative estimations of the respective concentrations are based on the measurements of responsiveness to gustatory antennal stimulation. We would expect that equal subjective rewards should result in similar acquisition functions and in similar memory formation in animals that differ in their sensory sensitivities.

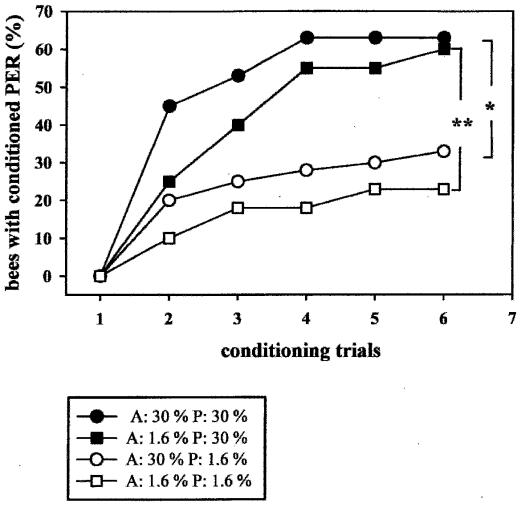

Figure 1. Tactile acquisition curves of bees that were stimulated with different sucrose concentrations at antenna and proboscis during conditioning. All of these bees had a uniform high gustatory responsiveness measured by antennal sucrose stimulations. The *x*-axis shows the number of conditioning trials. The *y*-axis gives the percentage of bees showing the conditioned proboscis extension response (PER). (A) Antennal stimulation. (P) Proboscis stimulation. The levels of acquisition differed significantly between bees that were stimulated with different sucrose concentrations at the proboscis and with equal sucrose concentrations at the antenna [(*) $P \leq 0.05$, (**) $P \leq 0.01$; two-tailed Fisher Exact Probability Test] but not between bees that were stimulated with different sucrose concentrations at the antenna, while the proboscis was stimulated with the same sucrose concentration ($P > 0.05$; two-tailed Fisher Exact Probability Test). In each group 40 bees were tested.

Results

Learning with different sucrose concentrations applied to antenna and proboscis

In this experiment, we wanted to quantify the effects of the sucrose concentration applied to the antennae and that applied to the proboscis on acquisition during tactile learning. As foraging bees differ in their responsiveness to gustatory antennal stimulation, we selected animals with uniform and high responsiveness for this experiment. Each of these bees responded to antennal stimulation with water, and all tested sucrose stimuli with proboscis extension. During conditioning, the animals were stimulated with either a low or a high sucrose concentration at the antenna and were subsequently stimulated at the proboscis with a high or a low concentration. Thus the effects of antennal and proboscis stimulation on acquisition during tactile learning could be separated.

Bees of all four groups showed significant acquisition. However, of bees that were stimulated with the same sucrose concentration at the antenna, those that were stimulated with 1.6% sucrose at the proboscis reached a significantly lower level of acquisition than the ones that were stimulated with 30% sucrose at the proboscis (Fig. 1; A: 1.6%, P: 1.6% vs. A: 1.6%, P: 30%; $P \leq 0.01$; A: 30%, P: 1.6% vs. A: 30%, P: 30%; $P \leq 0.05$; two-tailed Fisher Exact Probability Test). Bees that were stimulated with different sucrose concentrations at the antenna but with the same sucrose concentration at the proboscis did not differ significantly in their level of acquisition (A: 1.6%, P: 1.6% vs. A: 30%, P: 1.6%; $P > 0.05$; A: 1.6%, P: 30% vs. A: 30%, P: 30%; $P > 0.05$; two-tailed Fisher Exact Probability Test).

The overall degree of acquisition was measured as acquisition scores. These scores comprise the total number of conditioned responses of each individual during acquisition (see Materials and Methods). Acquisition scores differed significantly between groups of bees that were stimulated with different sucrose concentrations at the proboscis, but not between groups that had been stimulated with different concentrations at the antenna (KW = 24.77, $n = 40$ in each group, $P \leq 0.0001$; two-tailed Kruskal Wallis H Test). Of bees that were stimulated with the same sucrose concentration at the antenna, those that were rewarded with 1.6% sucrose at the proboscis had significantly smaller acquisition scores than bees that were stimulated at the proboscis with 30% sucrose (A: 1.6%, P: 1.6% vs. A: 1.6%, P: 30%; mean rank difference = 32.68, $P \leq 0.01$; A: 30%, P: 1.6% vs. A: 30%, P: 30%; mean rank difference = 34.38, $P \leq 0.01$; two-tailed Dunn's Multiple Comparison Test). Animals that were stimulated with the same sucrose concentration at the proboscis but were stimulated at the antennae with different sucrose concentrations did not differ significantly in their acquisition scores (A: 1.6%, P: 1.6% vs. A: 30%, P: 1.6%; mean rank difference = 9.53, $P > 0.2$; A: 1.6%, P: 30% vs. A: 30%, P: 30%; mean rank difference = 11.23, $P > 0.1$; two-tailed Dunn's Multiple Comparison Test). These results show that the concentration of the sucrose solution applied to the proboscis determined the level of acquisition in tactile learning for bees that had a similar gustatory responsiveness.

Gustatory responsiveness to antennal and to proboscis stimulation

The above experiment showed that the concentration of sucrose applied to the proboscis determines the level of acquisition in tactile antennal learning in bees that had a similar responsiveness to gustatory antennal stimulation. Earlier experiments had demonstrated that animals that differ in responsiveness to gustatory antennal stimulation also differ in tactile and olfactory acquisition (for review, see Scheiner et al. 2004). Bees with high

gustatory responsiveness reached higher levels of acquisition than bees with low responsiveness to gustatory antennal stimulation. These findings suggest that gustatory responsiveness measured by stimulating the antennae should correlate with that measured by stimulating the proboscis. This hypothesis was tested in the following experiment by measuring responsiveness to water and sucrose at antenna and proboscis in the same individuals.

Bees were more responsive to water and low sucrose concentrations when these were applied to the antenna than when they were applied to the proboscis (Fig. 2). For water and sucrose concentrations up to 3% sucrose, bees responded significantly more often after antennal stimulation than after proboscis stimulation (Fig. 2A; $P \leq 0.001$, $n = 92$; two-tailed Fisher Exact Probability Test). At higher sucrose concentrations, the concentration-response curves of antenna and proboscis converged. Gustatory response scores (GRSs) were used as a measure for overall responsiveness. They comprise the total number of PERs that a bee displays to water and all sucrose concentrations tested. The GRSs measured after gustatory antennal stimulation correlated significantly with those measured after gustatory proboscis stimulation ($\rho = 0.514$, $n = 92$, $P \leq 0.001$; Spearman rank correlation). These results demonstrate that responsiveness to gustatory antennal stimulation is an indicator of the responsiveness to gustatory proboscis stimulation.

The differences between responsiveness to gustatory antennal stimulation and that to gustatory proboscis stimulation were estimated by testing sigmoid regression models for the concentration-response curves. For these calculations, we only used the responses of bees with intermediate gustatory responsiveness, because bees belonging to these response groups (see below and Table 1) were used for conditioning (Fig. 2B). We found significant regressions for both behavioral parameters (Fig. 2B, responsiveness to gustatory antennal stimulation: $R^2 = 0.98$, df = 6, $P \leq 0.001$; responsiveness to gustatory proboscis stimulation: $R^2 = 0.95$, df = 6, $P \leq 0.01$). The inflection points (concentrations that elicit 50% responses) for both curves differ by ~1.2 log units, indicating that the sucrose concentrations that elicit 50% responses during antennal stimulation are about one order of magnitude smaller than the concentrations for proboscis stimulation. The relationships between responsiveness to gustatory antennal stimulation and that after gustatory proboscis stimulation can be used to estimate equal subjective reward concentrations for bees that differ in their responsiveness.

Estimation of equal subjective rewards

The main hypothesis of this study was that responsiveness to gustatory antennal stimulation is an indicator of the individual evaluation of a reward stimulus during associative learning. We assumed that it would be possible to reward bees displaying a different responsiveness to gustatory antennal stimulation with equal subjective rewards if we adjusted the concentration of sucrose in the rewarding stimulus accordingly. Our estimations of equal subjective reward concentrations were based on the following experimental findings:

1. Individual bees differ in their responsiveness to gustatory antennal stimulation, which correlates with their learning performance (for review, see Scheiner et al. 2004).
2. In learning experiments, the sucrose concentration applied to the proboscis is decisive for the learning success (Experiment 1, Fig. 1).
3. Responsiveness to gustatory antennal stimulation has a defined relationship with responsiveness to gustatory proboscis stimulation (Experiment 2, Fig. 2B).

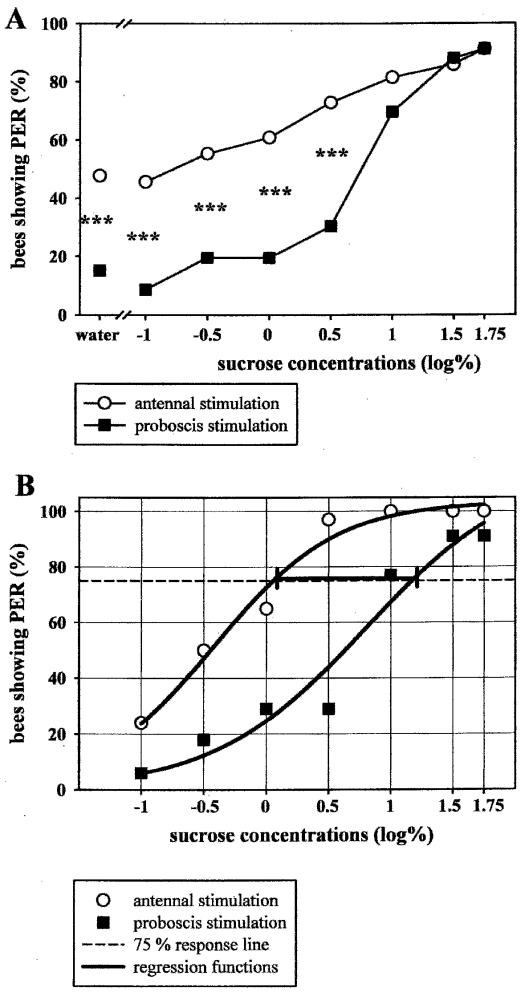

Figure 2. (*A*) Responses to water and increasing sucrose concentrations after stimulation of the antenna or the proboscis. The *x*-axis shows the water stimulation and the logarithm of the sucrose concentrations tested. The *y*-axis gives the percentage of bees showing the proboscis extension response (PER). The symbols represent the responses of the bees. Significant differences between responses after antennal stimulation and those after proboscis stimulation are indicated [(***) $P \leq 0.001$; two-tailed Fisher Exact Probability Test]. Here 92 bees were tested. (*B*) Sucrose-concentration response curves measured after gustatory stimulation of antenna and proboscis of bees with the same responsiveness as those that were conditioned. These bees belong to GRS classes 3 to 6, which are subgroups of the bees shown in *A*. The *x*-axis shows the logarithm of the sucrose concentrations tested. The *y*-axis gives the percentage of bees showing the proboscis extension response (PER). The symbols represent the responses of the bees. The broken line marks the 75% response criterion. The full lines show the sigmoid regression functions. The following sigmoid regression functions were found: for antennal stimulation, $f(x) = 103.52/\{1 + \exp[-(X + 0.41)/0.48]\}$; $R^2 = 0.98$; $P \leq 0.001$; for proboscis stimulation it is $f(x) = 116.02/\{1 + \exp[-(X - 0.80)/0.61]\}$; $R^2 = 0.95$; $P \leq 0.01$. The bar at the 75% criterion indicates the concentration difference that is necessary to evoke the 75% response during antennal and proboscis stimulation. Here 34 animals were tested.

To calculate equal subjective reward concentrations, we first measured responsiveness to antennal stimulation with water and to different sucrose concentrations in a large sample of bees (Fig. 3). Individuals were placed in response classes according to their GRSs. The response curves of bees in different GRS classes were similar and were shifted along the *x*-axis. For the estimation of the reward concentration, we used the sucrose-concentration response curves of the different GRS classes. From pilot studies we

Table 1. Sucrose concentrations of the rewards in associative tactile learning of bees with different gustatory responsiveness

GRS class	Estimated sucrose concentration (log %) for 75% response	Estimated subjective sucrose concentration (log %) for reward	Estimated subjective sucrose concentration (%) for reward
0	∞	∞	∞
1	1.4	2.4	251.2%
2	0.9	1.9	79.4%
3	0.5	1.5	31.6%
4	0.2	1.2	15.8%
5	−0.06	0.94	8.71%
6	−0.9	0.1	1.3%
7	Cannot be estimated as all responses are >75%	Cannot be estimated as all responses are >75%	Set at 31.6% → "control" group

Bees were placed in classes according to their antennal gustatory response scores (GRSs). From the concentration–response curves of bees in the different GRS classes, we estimated the sucrose concentration that elicited proboscis extension in 75% of the bees in each GRS class. The reward for each GRS class was chosen to be 1 log unit above this concentration (see text for details). Animals of GRS classes 0–2 were not conditioned because it was impossible to reward bees with the calculated reward concentrations. For GRS class 7 the theoretical 75% response concentration could not be estimated because all bees of this group responded to water and all tested sucrose concentrations. This group was used as the "control" group. Bees in GRS class 7 were rewarded with the same sucrose concentration as bees in GRS class 3 and therefore received a higher subjective reward compared to all other conditioned groups.

had rough estimations of the sucrose reward concentrations that induce significant associative learning in bees. We used the 75% response criterion (Fig. 3) for the estimation of concentrations that elicit the same responses in bees of different responsiveness to gustatory antennal stimulation (GRS classes). Sucrose concentrations for the 75% response criterion could be estimated for bees in the GRS classes 1 to 6. Animals that were very sensitive (GRS class 7) were always above the 75% criterion, while bees that were very insensitive (GRS class 0) never reached it. To estimate the sucrose concentrations of the reward, we used the sigmoid functions shown in Figure 2B. On average, the 75% criterion for proboscis stimulation was reached for concentrations that were ~1 log unit higher than those for antennal stimulation. We therefore decided to reward bees of the different GRS classes with sucrose solutions whose concentrations were 1 log unit above the sucrose concentrations for the 75% criterion during antennal stimulation.

For the conditioning experiment with equal subjective rewards, we could thus use bees of the GRS classes 3 to 6 (Table 1). The GRS classes 0 and 7 were excluded before. It was impossible to produce the necessary reward concentrations for the GRS classes 1 (251%) and 2 (79%) (Table 1). We used GRS class 7 as a control and rewarded bees in that group with the same absolute sucrose concentration as bees in GRS class 3 (Table 1). The goal was to test whether bees with different GRSs display a different learning performance when they are rewarded with the same absolute sucrose reward, as has been the case in earlier experiments (for review, see Scheiner et al. 2004).

Tactile learning of bees receiving equal subjective sucrose rewards

The course of acquisition was very similar in all groups of bees that received the same subjective reward strength (Fig. 4A), and the level of acquisition measured during the fourth conditioning trial did not differ between these groups (GRS classes 3 to 6, $P > 0.05$, two-tailed Fisher Exact Probability Test). In contrast, bees that received a higher subjective reward reached a significantly higher acquisition level (GRS class 7 vs. GRS class 3, $P \leq 0.001$, two-tailed Fisher Exact Probability Test). A nonparametric analysis of variance on the effects of GRS on acquisition scores of all groups revealed a significant effect of GRS (KW = 72.76; $P \leq 0.0001$; two-tailed Kruskal-Wallis H Test). Bees in GRS class 7, which had received a higher subjective reward than bees of all other GRS classes, also displayed significantly higher acquisition scores than bees in the other GRS classes ($P \leq 0.001$ for all comparisons between GRS class 7 and each of the other GRS classes; two-tailed Dunn's Test). The acquisition scores of bees receiving the same subjective reward (GRS classes 3 to 6) did not differ significantly ($P > 0.05$ for all comparisons, two-tailed Dunn's Test). This shows that there were basically no differences in the acquisition of bees that received equal subjective rewards, whereas bees that had received a higher subjective reward (GRS class 7) displayed a better acquisition.

Nevertheless, GRS correlated with acquisition scores in bees that received equal subjective rewards (GRS classes 3–6; $\rho = 0.18$, $P \leq 0.01$, Spearman rank correlation coefficient). This shows that minor but systematic differences in the degree of acquisition remained between the groups that had received equal subjective rewards.

In the retention test 24 h after conditioning, bees that had been rewarded with the same subjective reward did not differ in their response probability (Fig. 4B; $P > 0.05$, two-tailed Fisher Exact Probability Test). In contrast, bees that had received a higher subjective reward displayed a significantly higher response probability (GRS class 3 vs. GRS class 7; $P \leq 0.05$; two-tailed Fisher Exact Probability Test). In the subset of bees that survived until

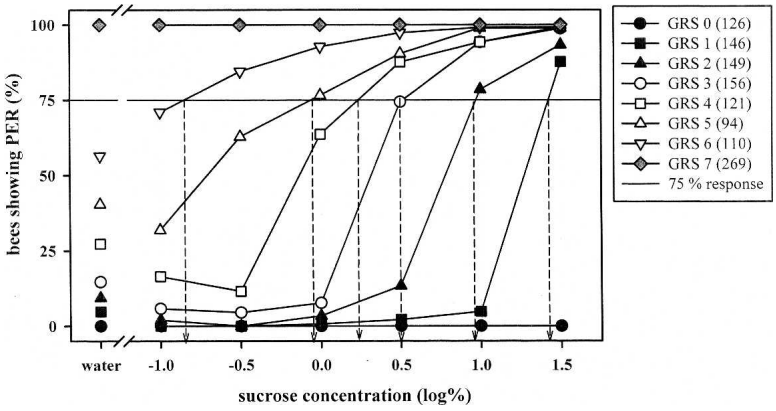

Figure 3. The percentages of proboscis responses during antennal stimulation with water and increasing sucrose concentrations of a large sample of bees. The *x*-axis shows the water stimulus and the logarithm of the sucrose concentrations applied to the antennae. The *y*-axis gives the percentages of bees showing the proboscis extension response (PER). Bees were placed in classes according to their gustatory response scores (GRSs) measured by stimulating the antennae. The number of bees tested in each GRS class is shown in parentheses. For the estimation of equal subjective rewards, the line for the 75% response criterion is indicated. The reward concentration at the proboscis was 1 log unit above the sucrose concentration for the 75% response in each GRS class. For details, see text.

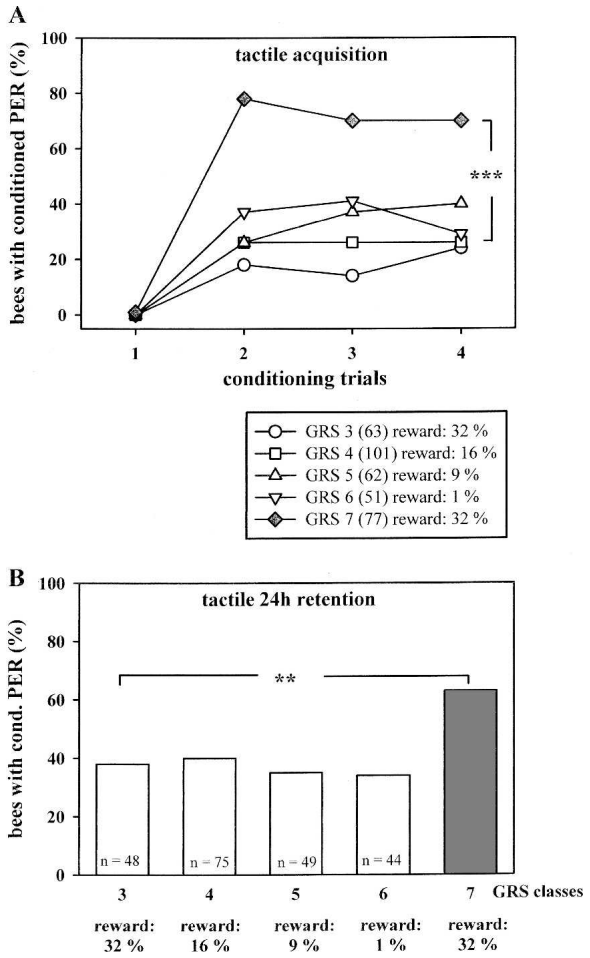

Figure 4. (*A*) Tactile acquisition curves of bees in different GRS classes. The *x*-axis shows the different acquisition trials. The *y*-axis shows the percentages of bees showing conditioned proboscis extension (PER). Individuals in GRS classes 3 to 6 (white symbols) received the same subjective rewards. They did not differ significantly in the acquisition level measured at the fourth trial. Bees in GRS class 7 (gray symbols), which had been rewarded with the same absolute reward concentration as bees in GRS class 3, reached a significantly higher acquisition level [(***) $P \leq 0.001$; two-tailed Fisher Exact Probability Test]. The numbers in parentheses in the legend indicate the number of bees tested in each group. (*B*) Tactile retention 24 h after conditioning in bees with different GRS. Only bees that survived 24 h are shown. Bees in GRS classes 3 to 6 (white columns) received the same subjective rewards, based on their GRSs. Individuals in GRS class 7 (gray column), in contrast, received the same absolute reward concentration as individuals in GRS class 3. They therefore received a higher subjective reward than bees in GRS class 3 and thus served as a control group. The figure shows the percentages of conditioned responses in each GRS class. The significant difference between bees in GRS class 7 and those in GRS class 3 is shown [(**) $P \leq 0.01$; two-tailed Fisher Exact Probability Test]. These groups had received different subjective rewards. All the groups that had received equal subjective rewards did not differ in their response probability. The sucrose concentrations of the rewards are shown for the different GRS classes. The number of bees tested is indicated.

the 48-h test, bees rewarded with equal subjective rewards did not differ in the 24-h test and in the 48-h test (Fig. 5; $P > 0.05$, two-tailed Fisher Exact Probability Test). Bees in GRS class 7, which had received a higher subjective reward than those in GRS class 3, had a significantly higher response probability in the 24-h retention test than bees in GRS class 3 (Fig. 5; $P \leq 0.01$; two-tailed Fisher Exact Probability Test). Bees in GRS class 7 no longer differed in their response probability 48 h after conditioning from those in GRS class 3 ($P > 0.05$; two-tailed Fisher Exact Probability Test).

Discussion

US evaluation and acquisition

The effect of US strength on learning is well established in learning theory (Annau and Kamin 1961; Rescorla and Wagner 1972; Lieberman 1993). Stronger USs initiate better learning and higher retention scores. Usually, US strength is related to some physical parameter. Animals are considered to be equally affected by this parameter, particularly in laboratory conditions in which animals belonging to the same test group are thought to respond equally to the stimuli. Individual differences even under fully controlled conditions are usually considered to be noise. The honeybee offers the unique opportunity to study the subjective component of US strength, because individuals differ in their responsiveness to sucrose solution, the US used in reward learning, even though they have been treated in the same way, have the same level of satiation, have been collected from the colony under identical conditions, and have been kept under similar conditions until trained and tested (Scheiner et al. 1999, 2001a,b,c, 2003). Pollen foragers, for example, are more responsive to the US sucrose than nectar foragers. But even within a sample of nectar foragers, individuals can differ widely in their antennal gustatory responsiveness. These individual differences in responsiveness to gustatory antennal stimulation correlate with differences in PER learning. Bees that display a high responsiveness to sucrose solutions when applied to the antennae generally reach higher levels of acquisition than bees with lower responsiveness to gustatory antennal stimulation if they can drink the same sucrose solution with the proboscis. This relationship between responsiveness to gustatory antennal stimulation and acquisition appears puzzling at first glance, because the sensory input that dominates acquisition is the concentration of the US sucrose applied to the proboscis and not to the antenna (Fig. 1). The explanation for this finding lies in the correlation of responsiveness to gustatory antennal stimulation and responsiveness to gustatory proboscis stimulation. Responsiveness to gustatory antennal stimulation is a reliable indicator of respon-

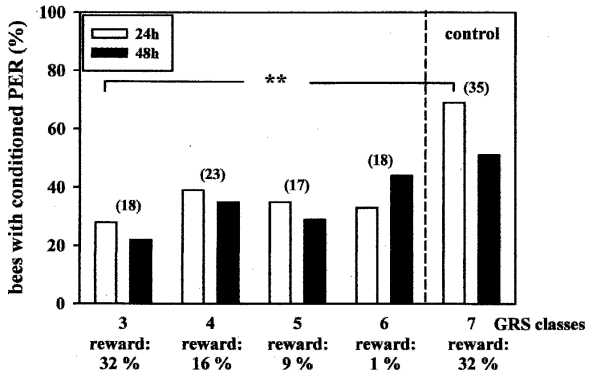

Figure 5. Percentages of bees responding with conditioned proboscis extension response (PER) 24 and 48 h after the last conditioning trial. Only animals that survived 48 h are displayed. The sucrose concentrations of the rewards are shown for the different GRS classes. The number of bees is given in parentheses. Bees of the GRS classes 3 to 6 had received the same subjective rewards. They did not differ significantly in their response probabilities. Bees in GRS class 7 had received a higher subjective reward, which was the same absolute reward as bees in GRS class 3 had received. At 24 h after conditioning, bees in GRS class 7 responded significantly more often than bees in GRS class 3 [(**) $P \leq 0.01$; two-tailed Fisher Exact Probability Test].

siveness to gustatory proboscis stimulation. Bees that are sensitive to sucrose stimuli applied to the antenna are also highly sensitive to stimuli applied to the proboscis. Bees in our earlier experiments (Scheiner et al. 1999, 2001a,b,c, 2003), which differed in their responsiveness to gustatory antennal stimulation, consequently also differed in their responsiveness to gustatory proboscis stimulation. We assume that the same sucrose solution that was used as reward had different subjective values for individuals differing in their responsiveness, and that therefore led to differences in tactile or olfactory acquisition.

Our current experiments demonstrate how responsiveness to gustatory antennal stimulation differs from responsiveness to gustatory proboscis stimulation (Fig. 2A). In bees of the GRS classes that were conditioned to the tactile object, the concentration-response curve of proboscis stimulation was shifted by ~1 log unit toward higher sucrose concentrations compared to antennal stimulation (Fig. 2B). Starting with the measurement of responsiveness to gustatory antennal stimulation, we can place each individual in a GRS class and estimate equal subjective reward concentrations for conditioning, which are then applied to antenna and proboscis.

Our experiments document that the responsiveness to gustatory stimuli correlates with the objective incentive value of the US. Equal subjective rewards lead to a similar learning performance in animals with a different responsiveness. This implies that the difference between the sucrose concentration used as reward and the individual response threshold to sucrose is decisive for the evaluation of a reward stimulus, as was suggested in Scheiner et al. (1999) under different experimental conditions. By keeping this difference equal between bees of different gustatory responsiveness, most of the differences in acquisition can be eliminated. These results are in accordance with the learning theory of Rescorla and Wagner (1972), which assumes that the strengths of the unconditioned and conditioned stimuli determine the strength of the association between the two stimuli, although in a different way. The US affects the plateau of acquisition, whereas the CS affects its steepness. This particular aspect will be addressed in a follow-up study. Here we demonstrate a way of estimating the strength of the US including differences between the sites of US sensing.

The covariance of responsiveness to gustatory stimulation of the antennae and proboscis suggests a common determinant controlling these and possibly other sensory and motor components of bee behavior (see also below). Such a determinant is likely to be implemented in a modulatory system and its specific wiring, but this system still needs to be described. There is evidence that responsiveness to gustatory or olfactory stimulation of the antennae is affected by the biogenic amines octopamine, tyramine, and dopamine (Mercer and Menzel 1982; Bicker and Menzel 1989; Menzel et al. 1990; Scheiner et al. 2002), but the interactions of these amines and the underlying signaling cascades are unclear.

The modulatory system underlying the reinforcing function of the sucrose reward in olfactory learning in the bee was found to be implemented in the ventral unpaired median neuron no. 1 in the maxillary neuromere (VUM_{mx1}), which is presumably octopaminergic (Hammer 1997). In addition, local octopamine injections immediately after olfactory stimulation into the antennal lobe or the mushroom bodies, the centers of associative learning in the honeybee, could replace the sucrose reward in olfactory learning (Hammer and Menzel 1998). It is thus likely that the specific wiring of octopamine-containing modulatory neurons provides the mechanistic basis for the reinforcing function of the sucrose reward.

In how far the response-releasing function of sucrose applied to antenna or proboscis and its reinforcing function during associative conditioning have common determinants is not clear. Both functions have genetic determinants (Scheiner et al. 2001a,b), but they are clearly dissociated in some respects. Stimulating the VUM_{mx1} neuron, for example, can substitute for the reinforcing function of sucrose in associative conditioning, but it does not release the PER as a sucrose stimulus that is presented to the antennae (Hammer and Menzel 1998). Injections of octopamine into bees depleted of biogenic amines by reserpine can restore the reinforcing function of sucrose during conditioning, but octopamine does not rescue the response-releasing function as measured in sensitization experiments (Menzel et al. 1999). In *Drosophila* larvae, it was shown that stimuli that strongly affect response-releasing behavior, such as the attractant fructose or the repellent quinine, do not necessarily affect olfactory choice behaviors and thus seem ineffective as reinforcers (Hendel et al. 2005).

In turn, some stimuli can act as reinforcers during conditioning, but they may not release a behavioral response. Fully satiated bees, for example, which do not show the PER at antennal stimulation with sugar, can nevertheless be conditioned associatively. When they are hungry again, they show the conditioned PER (Hammer and Menzel 1994). In associative olfactory or tactile learning, the reward strongly depends on the gustatory input via the proboscis (Fig. 1), whereas the PER is usually elicited by sucrose stimulation of the antenna. Finally, even the absence of a response-releasing stimulus can have inhibitory effects on associative learning such as was shown for negative patterning discrimination (Deisig et al. 2001).

US evaluation and retention

While the relationship between individual gustatory responsiveness and acquisition has been the focus of several studies, the role of gustatory responsiveness in retention has received little attention so far. Our experiments demonstrate that individual gustatory responsiveness also correlates with retention. Bees in GRS class 7, which were rewarded with the same sucrose concentration (32% sucrose) as those in GRS class 3, had a significantly higher proportion of conditioned responses in the 24-h retention test. Apparently, a good retention is related to high gustatory responsiveness during conditioning. In most learning experiments with bees, gustatory responsiveness has not been tested prior to conditioning, which can be related to a large performance variance in memory tests. If bees of GRS classes 3 and 7, for example, are used for a learning experiment, they will respond with PER when the antenna is stimulated with 32% sucrose. Without knowing the individual responsiveness, it is assumed that a reward of 32% sucrose will have the same incentive value for all the animals. This hypothesis can be rejected on the basis of our data.

Differences between the retention scores of bees in GRS class 3 and those of bees in GRS class 7 disappeared 48 h after training, when survival was lower than 24 h after conditioning, apparently because considerably fewer bees in GRS class 7 showed conditioned responses in the 48-h test (51% of the bees) compared to the 24-h test (69% of the bees). This phenomenon could be related to differences in a specific form of modulation, the "preparedness to learn," if we assume that responsiveness is also an indicator for motivation. An additional component could be sensitivity to extinction. We tested our bees in a cumulative way, which might have led to additive extinction. In the context of our results, the findings by Giurfa and Malun (2003) suggest that bees that initially differ in their responsiveness to the reward stimulus and thus in their "preparedness to learn" could also differ in their sensitivity to extinction. It is tempting to assume that one common factor, possibly the octopamine-containing

modulatory system mentioned above, leads not only to covariance of antennal and proboscis gustatory responsiveness, but is also related to a "preparedness to learn" and the resistance to extinction. It will be interesting to manipulate the octopamine system and to test whether the behavioral subsystems can be related to different anatomical compartments of this modulatory system.

Our experiments further show that gustatory responsiveness correlates positively with survival both 24 h after conditioning ($\rho = 0.152$, $n = 354$, $P = 0.004$; Spearman rank correlation) and 48 h after conditioning ($\rho = 0.147$, $n = 354$, $P = 0.005$; Spearman rank correlation). This relationship is independent of feeding state, because all bees were fed to satiation at the evening of each day and on the morning of the subsequent day. The performance in the 24-h test was not related to survival in a systematic way. These results support the assumption that individual gustatory responsiveness is a decisive indicator of the behavioral state of a bee. Performance in retention tests appears to be unrelated to survival, at least across the different GRS classes.

The impact of individual operant antennal scanning behavior

Although bees that received equal subjective rewards did not differ significantly in their acquisition level or acquisition scores, a correlation between GRSs and acquisition scores remained. This implies that differences in acquisition of individuals displaying a different gustatory responsiveness could not be completely compensated for by equalizing the subjective reward values. The remaining correlation between GRSs and acquisition scores could be related to factors that affect the perception and salience of the CS or the operant activity of the bees rather than the evaluation of the US. The operant antennal scanning behavior is a decisive component of tactile antennal learning, which could correlate with GRSs. For responsiveness to pollen, to olfactory stimuli and phototactic behavior correlations with gustatory responsiveness were shown (for review, see Scheiner et al. 2004). For that reason, we tested operant antennal scanning behavior in bees of high and those of low gustatory responsiveness. When a small tactile object such as was used in the tactile conditioning experiments was brought into the scanning range of bees with high gustatory responsiveness, the scanning frequency was significantly higher than in bees with low GRSs (Fig. 6; $t = 3.562$, $P \leq 0.01$, two-tailed t-test). We did not observe any obvious differences in antennal activity in bees with a different gustatory responsiveness when the antennae moved around spontaneously without touching an object. However, quantitative measurements of antennal movements in the absence of an object were not conducted. From numerous tactile learning experiments, we know that bees with high antennal scanning activity in the presence of an object learn the object faster than bees that only move their antennae slowly or reluctantly (J. Erber and R. Scheiner, pers. comm.). This implies that differences in the scanning activity of bees with different gustatory responsiveness might have been related to the correlation between GRSs and acquisition scores that remained despite the equal subjective rewards.

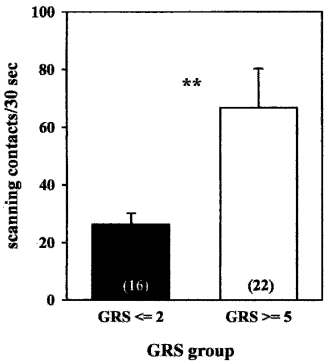

Figure 6. Contact frequencies of bees with low gustatory responsiveness (GRS $\leq$ 2, black column) and of bees with high gustatory responsiveness (GRS $\geq$ 5, white column) in the presence of a tactile plate. Mean contacts within the first 30 sec of object presentation and standard errors of the means are shown. The significant difference between the two groups is indicated [(**) $P \leq 0.01$, $t = 3.562$, two-tailed t-test]. The number of bees tested in each group is shown.

These findings and the correlations between gustatory responsiveness and responsiveness to other stimulus modalities imply that sensitivities for CS and US often correlate, which makes a distinction between the effects of CS strength and US strength on learning and memory very difficult. Our experiments demonstrate how the effects of different CS strengths can be compared by keeping the US value similar in animals that differ in their sensitivity. These findings could, for example, be used to test whether fine-scale differences in odor perception can contribute to differences in olfactory PER learning.

Our method of calculating equal subjective rewards for individuals with a different sensory sensitivity can also be adapted for other animal systems to separate the effects of CS strength and US strength on learning performance. However, it needs to be tested whether in other systems a similar relationship between sensitivity for CS or US and learning performance exists. In Wistar rats, for example, there seems to be no clear relationship between olfactory sensitivity and olfactory learning (Kramer and Apfelbach 2004).

Conclusions for foraging behavior

The relationship between individual gustatory responsiveness and learning behavior under laboratory conditions could be a decisive indicator of the foraging performance of bees under natural conditions. We know that bees that collect water or pollen have the highest gustatory responsiveness, whereas bees collecting nectar or both pollen and nectar are less responsive to water and sucrose (Page et al. 1998; Scheiner et al. 1999; Pankiw and Page 2000; Scheiner et al. 2001b). If our findings also apply to the foraging behavior of these bees, we assume that the relationship between gustatory responsiveness and learning behavior is directly related to the collection of different materials. Pollen collectors do not get high sucrose rewards during their foraging trips, and water foragers do not receive any sucrose rewards at all. Both groups of foragers should therefore accept water (in a water pond or dew on a pollen bearing flower) as reward. Most pollen and water collectors usually display a gustatory responsiveness that is comparable to that of bees in GRS classes 6 or 7 in our experiment. Because in our experiment bees in GRS class 6 learned as well as bees in the other groups, although they were only rewarded with 1% sucrose, it is conceivable that a water reward suffices for pollen or water collectors for efficient learning. Nectar foragers, on average, are less responsive to water and sucrose and generally display a wider distribution of GRSs than pollen foragers (Page et al. 1998; Scheiner et al. 1999, 2001b; Pankiw and Page 2000). This wider distribution of GRSs might ensure that nectar foragers exploit nectar sources of high profitability, but will also collect nectar of lower quality when nothing else is available. These assumptions need to be tested with foragers under free-flying conditions. We already have some evidence that tactile learning under free-flying conditions is comparable to that under laboratory conditions (Erber et al. 1998) and that nectar bees with higher gustatory responsiveness collect more diluted nectar than those with lower responsiveness (Pankiw and Page 2000).

These findings and results from other laboratories on the role of sensory stimuli in learning and foraging (Greggers and Menzel 1993; Giurfa 1996; Ben Shahar et al. 2000; Chandra et al. 2000; Masterman et al. 2000; Sandoz et al. 2001; Barron et al. 2002) provide us with a solid experimental basis for a detailed analysis of the multiple factors contributing to the processing of sensory stimuli, learning, and foraging behavior in honeybees.

Materials and Methods

Preparation of bees

For all experiments reported here we used non-pollen foragers. This group of foragers is extremely useful for studies on the relevance of sucrose responsiveness for different behaviors, because non-pollen foragers are particularly diverse in their responsiveness to sucrose solutions throughout the year (Scheiner et al. 2003). Returning bees were individually caught at the entrance of their hive and cooled in a refrigerator maintained at +7°C until they showed first signs of immobility. They were individually fixed in small tubes with a strip of adhesive tape between head and thorax and another strip of tape over the abdomen. To measure proboscis responses during stimulation of the proboscis with water or sucrose, we used plastic holding tubes with a rim on which the proboscis was placed. With these holders the proboscis of a bee could be stimulated with a liquid and the proboscis extension response could be observed at the same time. For the other experiments, we used metal tubes as described in Erber et al. (1998).

Measuring gustatory responsiveness at antenna and proboscis

Gustatory responsiveness for antennal stimuli was tested in each individual bee used in these experiments. Bees that were conditioned in the course of the experiment had their eyes occluded with black paint, while the eyes were not occluded in animals that were not conditioned. We did not observe any differences in the responsiveness of animals with open or occluded eyes.

In animals that were later conditioned, gustatory responsiveness was measured in each individual 1 h after mounting in the tube by using the proboscis extension response (PER). The antennae of each bee were first stimulated with a droplet of water and then with the following sucrose solutions offered in ascending order: 0.1%, 0.3%, 1%, 3%, 10%, and 30%. The inter-stimulus interval was ~2 min to exclude sensitization effects. The total number of proboscis responses following the seven different solutions constitutes the gustatory response score (GRS) of an individual (for review, see Scheiner et al. 2004). This score is a measure for the gustatory responsiveness of an individual. Bees with high GRSs were very responsive to water and sucrose. Those with low GRSs displayed a low gustatory responsiveness. After testing their responsiveness, each bee was placed into one of the GRS classes 0–7, according to its GRS.

The relationships between antennal and proboscis stimulation were measured in a separate group of animals. In this experiment, proboscis extension was tested during stimulation of the antennae or the proboscis with the seven solutions. To exclude any bias due to the sequence of stimulations, 50% of the bees were first stimulated at the antennae and then at the proboscis, and 50% of the animals were stimulated in the reverse order. For graphic display (Fig. 2), the percentage of bees showing PER at antennal or proboscis stimulation with water and different sucrose concentrations was calculated. Statistical comparisons between antennal and proboscis responses were done using two-tailed Fisher Exact Probability Tests (Graph Pad Instat 3.0).

The concentration-dependent proboscis responses for antennal or proboscis stimulation can be approximated by the sigmoid regression function

$$f = a/(1 + \exp[-(X - X_0)/b])$$

in which a, b, and X_0 are parameters of the function and X is the logarithm of the sucrose concentration (log %). The inflection point X_0 indicates the stimulus concentration that elicits 50% responses. The parameters of the nonlinear regression functions for antennal and proboscis stimulation were estimated using the regression module of the Sigma-Plot 2001 software.

Response scores of antennal stimulation or of proboscis stimulation were calculated as described above. These response scores represent the total number of PERs to stimulation with water and the six sucrose concentrations at either stimulation site. These scores could range from 0 (no response) to 7 (PER at stimulation with water and all tested sucrose concentrations). Correlations between scores for antennal stimulation and proboscis stimulation were tested using Spearman rank correlation coefficients.

Tactile learning with different sucrose concentrations at antenna and proboscis

It was the objective of this experiment to test the effects of different sucrose concentrations applied to the antennae and the proboscis during tactile learning. The eyes of the bees were occluded with black paint to block visual inputs (Erber et al. 1998). For this experiment, it was essential to use bees with identical sensory responsiveness. Therefore, only bees with a high responsiveness (GRS class 7) after gustatory antennal stimulation were conditioned. These bees responded with proboscis extension to antennal stimulation with water and all tested sucrose solutions.

Before conditioning started, bees were tested for their spontaneous responses to the tactile object, which consisted of a 3 × 4-mm metal plate with vertical engravings (Erber et al. 1998). The plate was brought into the scanning range of the bee antennae, and it was tested whether the bee responded with proboscis extension during scanning the plate. If the bee responded spontaneously, it was discarded from the further experiment. The rate of spontaneous responses was very low and was therefore not analyzed statistically. Each bee was then conditioned six times to the tactile stimulus, the inter-trial interval being 5 min. During conditioning, the plate was brought into the scanning range of the bee antennae. After scanning the plate for ~3 sec, the proboscis extension response was elicited by applying sucrose solution to the antennae. Once the bee extended its proboscis, it was allowed to lick at the sucrose droplet for 1 sec, before it was removed. Shortly afterward, the plate was removed from the scanning range of the bee. During each conditioning trial, it was recorded whether the bee showed conditioned proboscis extension during the presentation of the plate.

For conditioning, bees were split into four different groups that were stimulated with different sucrose concentrations at antenna and proboscis. As only animals with high responsiveness were used for these experiments, all animals responded with PER to each of the tested stimulus concentrations. The following four stimulus combinations were used:

1. stimulation with 1.6% sucrose at proboscis and with 1.6% sucrose at antenna;
2. stimulation with 1.6% sucrose at proboscis and with 30% sucrose at antenna;
3. stimulation with 30% sucrose at proboscis and with 1.6% sucrose at antenna;
4. stimulation with 30% sucrose at proboscis and with 30% sucrose at antenna.

For graphic presentation, we calculated for each group the percentage of bees showing conditioned proboscis extension responses in the six acquisition trials. The level of acquisition was compared between pairs of groups using two-tailed Fisher Exact Probability Tests. To analyze and compare the overall differences in acquisition between the four groups, the number of conditioned responses displayed in the six acquisition trials was calculated for each individual. This "acquisition score" of an individual represents the sum of conditioned responses and is a good measure for acquisition (Scheiner et al. 1999, 2001a,b,c, 2003). As

the animals were conditioned six times, the acquisition score has a range between 0 and 6. The effect of different sucrose concentrations applied to antenna and proboscis on acquisition scores was tested using a two-tailed Kruskal Wallis H Test, because the data were not distributed normally. The Dunn's Multiple Comparison Test was used as a post hoc test (GraphPad Instat 3.0).

Estimating equal subjective rewards for bees with different GRSs

The proboscis responses of large numbers of bees (between 94 and 269 animals per group) at stimulation with different sucrose concentrations were used to estimate average concentration-response curves for the different GRS classes (Fig. 3). The sucrose concentrations that elicit PER in 75% of the animals during antennal stimulation can be estimated graphically in each GRS class (Fig. 3). The concentration that elicits proboscis extension in 75% of the bees during proboscis stimulation is on average ~1 log unit higher than the equivalent concentration for antennal stimulation with sucrose (Fig. 2B). The equal subjective reward concentration for each individual was determined by measuring the responsiveness after gustatory antennal stimulation, which defines the GRS class of an animal. Individuals in GRS classes 3 to 6 received a sucrose reward whose concentration was 1 log unit higher than the sucrose concentration that elicited PER in 75% of the bees after antennal sucrose stimulation (Table 1). Only bees in GRS class 7 received a higher subjective reward. They served as the control group and received the same sucrose reward as bees in GRS class 3.

Tactile learning of bees receiving equal subjective sucrose rewards

Bees were collected and mounted, their GRSs were measured, and animals in the appropriate GRS classes were prepared for conditioning. Each bee was conditioned four times to a small tactile plate. The interval between conditioning trials was 10 min. The sucrose concentrations of the rewards for bees in each GRS class are shown in Table 1. Bees were conditioned as described above. After the final conditioning trial (trial 4), individuals were placed in a humid chamber where they were kept till the retention tests started on the next days. They were fed to satiation at the end of a day and on the morning of the subsequent day with a 50% sucrose solution. At 24 h and at 48 h after conditioning, they were tested for their conditioned responses.

The conditioned responses before the fourth conditioning trial and 24 h or 48 h after the fourth trial were compared between different GRS classes using two-tailed Fisher Exact Probability Tests (Graph Pad Instat 3.0). The relationship between GRS and acquisition scores was analyzed using a two-tailed Kruskall Wallis H Test, because acquisition scores did not follow normal distributions. Dunn's Multiple Comparisons Tests were used as post hoc tests (GraphPad Instat 3.0). Spearman rank correlation coefficient tests were used to analyze the relationships between acquisition scores and GRS classes and between GRS, performance in the retention tests, and survival (SPSS 12.0). All tests were two-tailed. The significance level was 5%.

Tactile scanning frequency in bees with different GRSs

In this experiment, we used bees with different GRSs and placed them in front of a tactile plate. Antennal contacts with the plate were recorded in a Faraday cage. The bee was connected to reference potential by insertion of a chlorinated silver wire into its head capsule. The plate and the bee were enclosed by a transparent plastic humid chamber. The metal plate was connected to the input of an electrophysiological amplifier (EXT-01C, npi; Tamm). Contacts of the antenna with the plate caused a positive voltage deflection due to the transepithelial potential of the antenna. This DC-coupled signal was amplified (DPA-2F, npi) for detection of contacts using a window discriminator (WD-01, npi). The resulting TTL pulses were sampled with a CED 1401 laboratory interface (Cambridge Electronic Design) attached to a PC-AT-compatible host computer using the "Spike 2" software (Cambridge Electronic Design). Data were evaluated as the number of antennal contacts per 30 sec.

Because the scanning contacts for the different GRS groups followed normal distributions, the two-tailed *t*-test was used to compare the mean scanning frequencies of the two GRS groups.

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